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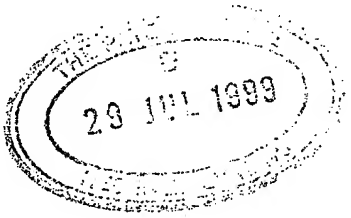

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Cardiff Road
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1. Your reference — C1092.00/C

2. Patent application number *(The Patent Office will fill in this part)* — **9917813.9** — 29 JUL 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 6048755002

If the applicant is a corporate body, give the country/state of its incorporation — England

4. Title of the invention

Improvements in or Relating to
RNA Polymerase Promoters

5. Name of your agent *(if you have one)* — Keith W Nash & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Cambridge CB2 1DP

Patents ADP number *(if you know it)* — 1206001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Keith W Nash & Co Date 29/07/1999

12. Name and daytime telephone number of person to contact in the United Kingdom M J LIPSCOMBE (01223) 355477

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C1092.00/C

Title: Improvements in or Relating to RNA Polymerase Promoters

Field of the Invention

This invention relates to nucleic acid complexes comprising a functional RNA polymerase promoter, and to a method of detecting a target nucleic acid sequence of interest.

Background of the Invention

RNA polymerases are enzyme molecules well-known to those skilled in the fields of molecular biology and molecular diagnostic kits. RNA polymerases synthesise RNA molecules from a DNA template strand.

Much research has been carried out on RNA polymerases, especially bacteriophage RNA polymerases. Generally, bacteriophage RNA polymerases are exceptionally active for *in vitro* transcription. This high level activity may be due in part to the fact that they are composed of a single polypeptide chain and do not require a dissociating initiation factor. These polymerases have been shown to be more active on supercoiled templates although they are also very active on linear templates (Smeekens & Romano 1986 Nucl. Acids Res. **14**, 2811).

Specifically, the RNA polymerase from the bacteriophage T7 has been shown to be very selective for specific promoters that are rarely encountered in DNA unrelated to T7 DNA (Chamberlin *et al*, 1970 Nature **228**, 227; Dunn & Studier 1983 J. Mol. Biol. **166**, 477). T7 RNA polymerase is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. T7 RNA polymerase is a highly active enzyme that transcribes about five times faster than does *Escherichia coli* RNA polymerase (Studier *et al*, 1990 Methods Enzymol. **185**, 60). The synthesis of small RNAs using T7 RNA polymerase has been described whereby sequences around the RNA polymerase promoter sequence are shown to be important in the reproducible improvement of yield of RNA produced (Milligan & Uhlenbeck, 1989 Methods Enzymol. **180**, 51 and Milligan *et al*, 1987 Nucl. Acids Res. **15**, 8783-8798). Other RNA polymerases that have similar

properties to T7 include those from bacteriophage T3 and SP6, the genes for which have all been cloned and the corresponding enzymes are commercially available. The optim promoter sequences for T7, T3 and SP6 polymerases are known, and are essentially 1 nucleotides long.

A number of methods have been disclosed, which utilise RNA polymerases to synthesise RNA directly or indirectly as the result of the presence of a particular nucleic acid sequence of interest ("target"). The presence of RNA (detected directly or indirectly) thus serves to signal the presence of the sequence of interest and can be used as the basis of assay methods and/or diagnostic methods or kits. Examples include the disclosures of WO 93/06240, WO 94/29481, EP 0851033, and EP 0552931.

Summary of the Invention

In a first aspect the invention provides a nucleic acid complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that third probe is hybridised to the arm region of the first probe adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

Preferably the sequence of the probes and the hybridisation reaction conditions are selected such that the first, second and third probes cannot become hybridised in the absence of the

target, such that formation of the RNA polymerase promoter occurs in a target-dependent manner.

In a second aspect, the invention provides a method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

The method of the second aspect of the invention thus results in formation of the complex of the first aspect.

It is an essential feature of the invention that the first and second probes, when hybridised to the target sequence, are adjacent or substantially adjacent to each other. Use of the term "adjacent" is herein intended to mean that there are no nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the complementary sequence of the probes. This proximity between the probes enables the complementary arm portions of the probes to anneal. As will readily be apparent to those skilled in the art, by designing the probes so as to allow for annealing to each other at greater separations from the target sequence, gaps may be introduced between the loci in the target nucleotide sequence to which the probes hybridise. In this

situation the probes are said to be "substantially adjacent", because there may be some nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the probes. Clearly, the number of intervening un-paired nucleotides of the target sequence can vary according to the design of the probes. Thus whilst it is preferred that the first and second probes hybridise so as to be adjacent, the probes may be separated by up to 5 nucleotides of target sequence, and the term "substantially adjacent" is intended to refer to such situations.

It will be apparent to those skilled in the art that the order of addition of probes in the formation of the nucleic acid complex is not critical: the third probe may, for example, be hybridised to the first probe before the second probe and sample are added. Alternatively, for example, all three probes may be simultaneously mixed with the sample containing the target molecule.

It will be further apparent to those skilled in the art that the second and third probes, which jointly provide one of the strands of the RNA polymerase promoter, are not covalently joined and the promoter sequence thus contains a "nick" in the phosphodiester backbone of one of the strands. As far as the inventors are aware, it has not been appreciated hitherto that such discontinuous nucleic acid strands could still give rise to a functional RNA polymerase promoter.

Preferred promoters for use in the invention are those recognised by bacteriophage polymerases, especially those promoters recognised by one of T3, T7 or SP6 polymerase. These will generally comprise a minimum of 17 bases, essentially double-stranded. The sequence of the double-stranded T3 RNA polymerase promoter (described in the prior art) is:

5' AAATTAACCCTCACTAAA 3'
3' TTTAATTGGGAGTGATTT 5'

(A number of variant T3 promoter sequences are also known, especially those in which

3', rather than AAA.)

The sequence of the T7 RNA polymerase promoter (described in the prior art) is:

5' TAATACGACTCACTATA 3'
3' ATTATGCTGAGTGATAT 5'

The sequence of the SP6 RNA polymerase promoter (described in the prior art) is:

5' ATTTAGGTGACACTATA 3'
3' TAAATCCACTGTGATAT 5'

One of the strands is provided by the first probe. Typically this will be the "sense" (+) strand, which is transcribed by the polymerase. Accordingly the first probe will generally comprise a stretch of "template" nucleic acid to be transcribed. The template will desirably comprise sequences which facilitate capture (e.g. hybridisation) of the resulting RNA transcript and/or detection. In addition, the first probe may desirably contain sequences (e.g. a "+12 sequence") 5' of the promoter sequence, which serve to increase the activity of the promoter. Specific instances of such sequences are disclosed in the examples below.

The inventors have elucidated the optimum sequence of +12 regions for the T7 polymerase (discussed in greater detail below) - it is not known at present if these are also optimum for, say, T3 and SP6 polymerases. If, as is possible, SP6 and T3 polymerases have different optimum +12 regions, it would be a simple matter for the person skilled in the art to identify the relevant sequence by trial-and-error, with the benefit of the present disclosure.

The sequences of preferred +12 regions, for inclusion in the template portion of the first probe, (in respect of T7 polymerase) are shown below in Table 1. The most active +12 region (giving greatest transcription) is at the top, with the other sequences shown in decreasing order of preference.

The destabilizing moiety is a chemical entity which is generally unable to undergo base pairing and hydrogen bonding in the normal manner as usually occurs wh complementary strands of nucleic acid become hybridised.

All manner of molecules may be suitable for use as a destabilizing moiety, although some compounds are specifically preferred, as described below. With the benefit of the present specification, the person skilled in the art will be able to test other compounds and readily select those which confer the appropriate degree of destabilization so as to prevent the hybridization of probes in the absence of target nucleic acid of interest. Particularly preferred, as a matter of convenience, are those compounds which are commercially available in a form (e.g. as phosphoramidites) which facilitates their incorporation into synthetic oligonucleotides using conventional automated solid phase nucleic acid synthesisers.

Destabilizing moieties which cannot base pair, but which nevertheless are capable of forming flexible folds and/or hairpin structures, are especially suitable. One such preferred destabilizing moiety comprises hexaethylene glycol (abbreviated herein as "Hex") (see Figure 5), which may be present singly or in tandem up to n times (where n can be any number ≥ 1, but conveniently has a maximum value of 5). In a particularly preferred embodiment, the third probe comprises one Hex molecule, where the number of bases opposite the destabilising moiety in the arm region of the first probe should be three to four bases. An alternative preferred destabilizing moiety comprises a plurality of alkylene (especially methylene) repeats. Particularly preferred are penta- or hexa-methylene spacers.

Other, less preferred, destabilizing moieties may alternatively be used. These include, but are not limited to, inosine, Virazole™ (N[1]-[1-β-D ribofuranosyl] 3-carboxamido-1,2,4,-triazole), Nebularin™ (N[9]-[1-β-D ribofuranosyl]-purine), nitropyrrole, ribose, propyl or combinations of the above eg. propyl-Hex-propyl, propyl-Hex-Hex-propyl, etc. Propyl may be replaced by, for example, ethyl, butyl, pentyl, heptyl, octyl etc. The number of bases opposite the destabilizing moiety in the arm region of the reciprocal probe should be x, where x is ≥ 1. The exact number of bases will of course depend on the size of the

destabilizing moiety and the value of n.

The following may be used as a guide: for each Hex molecule in the destabilizing moiety, the opposite probe should preferably comprise 3-4 bases (preferably 3); for each other molecule or radical mentioned above present in the destabilizing moiety, the opposite probe should preferably comprise a single base, with the exception of the following: butyl - two bases, pentyl - two bases, heptyl - three bases, and octyl - four bases.

The chemicals described above and used as destabilizing moieties are all commercially available (e.g. from Glen Research, USA).

The person skilled in the art will appreciate how to select appropriate conditions, materials and sequences for the probes, in order to ensure that the complex of the first, second and third probes (and hence formation of the functional RNA promoter) occurs in a target dependent manner. In essence, the degree of complementarity between the arm regions of the first and second probes must be such that, in the conditions employed, they will not hybridise unless stabilised by hybridisation of the respective foot regions of the first and second probes to the target.

Generally therefore, the foot regions of the first and second probes will comprise at least 10 bases, preferably at least 20 bases, and more preferably at least 25 bases. There is no upper limit on the size of the foot regions (which may, for example, comprise several kilobases). However, in practice, the probes will normally be *in vitro* synthesised oligonucleotides and so it will be preferred for the foot regions to comprise no more than about 75 bases.

In contrast, the number of complementary bases between the arm regions of the first and second probes will normally be no more than 25, typically less than 15, and optimally between 5 and 13 bases.

In preferred embodiments, the invention provides a method of generating a signal in a target-dependent manner (i.e. creation of the complex and hence formation of the

functional promoter) and causing amplification of this signal (generation of multiple RNA transcripts under the control of the promoter) in a system which may require the use of a single enzyme type (RNA polymerase), without the need for additional enzymes (e.g. DNA polymerases) to bring about the amplification. This is significant as the reaction conditions for optimum activity of RNA and DNA polymerases are generally mutually exclusive.

Detection Methods

RNA produced in accordance with the invention could be detected in a number of ways, preferably following amplification (most preferably by means of an isothermal amplification step e.g. as disclosed in US 5,399,491 and US 5,480,784). For example, newly-synthesised RNA could be detected in a conventional manner (e.g. by gel electrophoresis), with or without incorporation of labelled bases during the synthesis.

Alternatively, for example, newly-synthesised RNA could be captured at a solid surface (e.g. on a bead, or in a microtitre plate), and the captured molecule detected by hybridisation with a labelled nucleic acid probe (e.g. radio-labelled, or more preferably labelled with an enzyme, chromophore, fluorophore and the like). Preferred enzyme labels include horseradish peroxidase and alkaline phosphatase.

One preferred detection method involves the use of molecular beacons or the techniques of fluorescence resonance energy transfer ("FRET"), delayed fluorescence energy transfer ("DEFRET") or homogeneous time-resolved fluorescence ("HTRF"). Molecular beacons are molecules which a fluorescence signal may or may not be generated, depending on the conformation of the molecule. Typically, one part of the molecule will comprise a fluorophore, and another part of the molecule will comprise a "quencher" to quench fluorescence from the fluorophore. Thus, when the conformation of the molecule is such that the fluorophore and quencher are in close proximity, the molecular beacon does not fluoresce, but when the fluorophore and the quencher are relatively widely-separated, the molecule does fluoresce. The molecular beacon conveniently comprises a nucleic acid molecule labelled with an appropriate fluorophore and quencher.

One manner in which the conformation of the molecular beacon can be altered is by hybridisation to a nucleic acid, for example inducing looping out of parts of the molecular beacon. Alternatively, the molecular beacon may initially be in a hair-pin type structure (stabilised by self-complementary base-pairing), which structure is altered by hybridisation, or by cleavage by an enzyme or ribozyme.

FRET (Fluorescence Resonance Energy Transfer) occurs when a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced and the acceptor fluorescence is increased or sensitised.

Another approach (DEFRET, Delayed Fluorescence Energy Transfer) has been to exploit the unique properties of certain metal ions (Lanthanides e.g. Europium) that can exhibit efficient long lived emission when raised to their excited states (λexcitation = 337 nm, λemission = 620 nm). The advantage of such long lived emission is the ability to use time resolved (TR) techniques in which measurement of the emission is started after an initial pause, so allowing all the background fluorescence and light scattering to dissipate. Cy5 (Amersham Pharmacia) (λexcitation = 620 nm, λemission = 665 nm) can be used as the DEFRET partner.

HTRF (see WO92/01224; US 5,534,622) occurs where the donor (Europium) is encapsulated in a protective cage (cryptate) and attached to the 5' end of an oligomer. The acceptor molecule that has been developed for this system is a protein fluorophore, called XL665. This molecule is linked to the 3' end of a second probe. This system has been developed by Packard.

In another embodiment, the newly-synthesised RNA, before or after amplification, results in formation of a ribozyme, which can be detected by cleavage of a particular nucleic acid substrate sequence (e.g. cleavage of a fluorophore/quencher dual-labelled oligonucleotide).

The invention will now be further described by way of illustrative example and with

reference to the accompanying drawings, wherein:

Figures 1 and 3 are schematic representations of a nucleic acid complex in accordance with the invention;

Figures 2 and 4 are bar charts showing amount of RNA produced (in femtomoles) from various nucleic acid complexes, and;

Figure 5 is a schematic representation of a preferred destabilizing moiety for use in some embodiments of the invention.

Example 1

This example demonstrated that T7 RNA polymerase and three oligonucleotide probes could be used to detect a specific target sequence. Probe 1 contained a 34 base foot region complementary to the target sequence; an 8 base overlap sequence; the 17 base template strand of the T7 promoter; a +12 sequence and a capture and probe sequence for detection. Probe 2 comprised a 31 base foot region complementary to the target sequence; an 8 base overlap sequence and the first three bases of the complementary strand of the T7 promoter. Probe 3 contained the remaining 14 bases of the complementary strand of the 17 base T7 promoter sequence. The example is illustrated schematically in Figure 1.

1.1 **Preparation of oligonucleotides**

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

1.2 **Synthesis of RNA off hybridised oligonucleotides**

Hybridisation was achieved in an assay mixture that contained 10 fmol probe 1, 50 fmol

of probe 2, 25 fmol of probe 3 and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl at final concentration). The reaction volume was made up to 20 μl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C/second) to 37°C. T7 RNA polymerase (25 units) and 2 μl NTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)) were added and the mixture incubated at 37°C for 3 hours.

1.3 Capture and detection of synthesised RNA

20 μl of assay sample was added to 145 μl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavidin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 2.

1.4 List of oligonucleotides

Probe 1

5' TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGGCTCTCTCTCCCTA TAGTGAGTCGTATTAATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGA AGCG 3' (phosphate blocked)

Probe 2

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTTCGAAATTAA 3'

Probe 3

5' TACGACTCACTATA 3'

Probe 4 (target)

5' GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC
CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACTGAGA
ACAGAATGAAATTCTTC 3'

Sequence of transcribed RNA

5' GGGAGAGAGAGCCACAUCGGGUGAUAUCCAGAACGGAGACAAGGAG
GCA 3'

Capture Probe

5' TCTGCTGCCTGCTTGTCTGCGTTCT 3'

Detection probe (Wallac-AP)

5' GGATATCACCCG 3' (3' alkaline phosphatase labelled)

Example 2

This example demonstrated that a Hexaethylene glycol linker (Hex) positioned 3 bases from the 3' end of Probe 3 increased the amount of signal obtained. The example is illustrated schematically in Figure 3.

2.1 **Preparation of oligonucleotides**

All oligonucleotide probes were prepared and functionalised as described in Example 1.1. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. All oligonucleotides were HPLC purified using standard techniques.

2.2 **Synthesis of RNA off hybridised oligonucleotides**

Hybridisation was achieved in an assay mixture that contained 10 fmol probe 1, 50 fmol of probe 2, 50 fmol of probe 3 or probe 5 and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl at final concentration). The reaction volume was made up to 20 μl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 or probes 1, 2 and 5 without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids,

then cooled (by ramping at 0.1°C / second) to 37°C. T7 RNA polymerase (25 units) and 2 μl NTP mix (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)) were added and the mixture incubated at 37°C for 3 hours.

2.3 Capture and detection of synthesised RNA

20 μl of assay sample was processed as described in Example 1.3. Results are presented in Figure 4.

2.4 List of oligonucleotides

Probe 1

5' TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGGCTCTCTCTCCCTA TAGTGAGTCGTATTAATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGA AGCG 3' (phosphate blocked)

Probe 2

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTTCGAAATTAA 3'

Probe 3

5' TACGACTCACTATA 3'

Probe 4 (target)

5' GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACTGAGA ACAGAATGAAATTCTTC 3'

Probe 5 H = Hexaethylene glycol

5' TACGACTCACTHATA 3'

Sequence of transcribed RNA

5' GGGAGAGAGAGCCACAUCGGGUGAUAUCCAGAACGGAGACAAGGAG GCA 3'

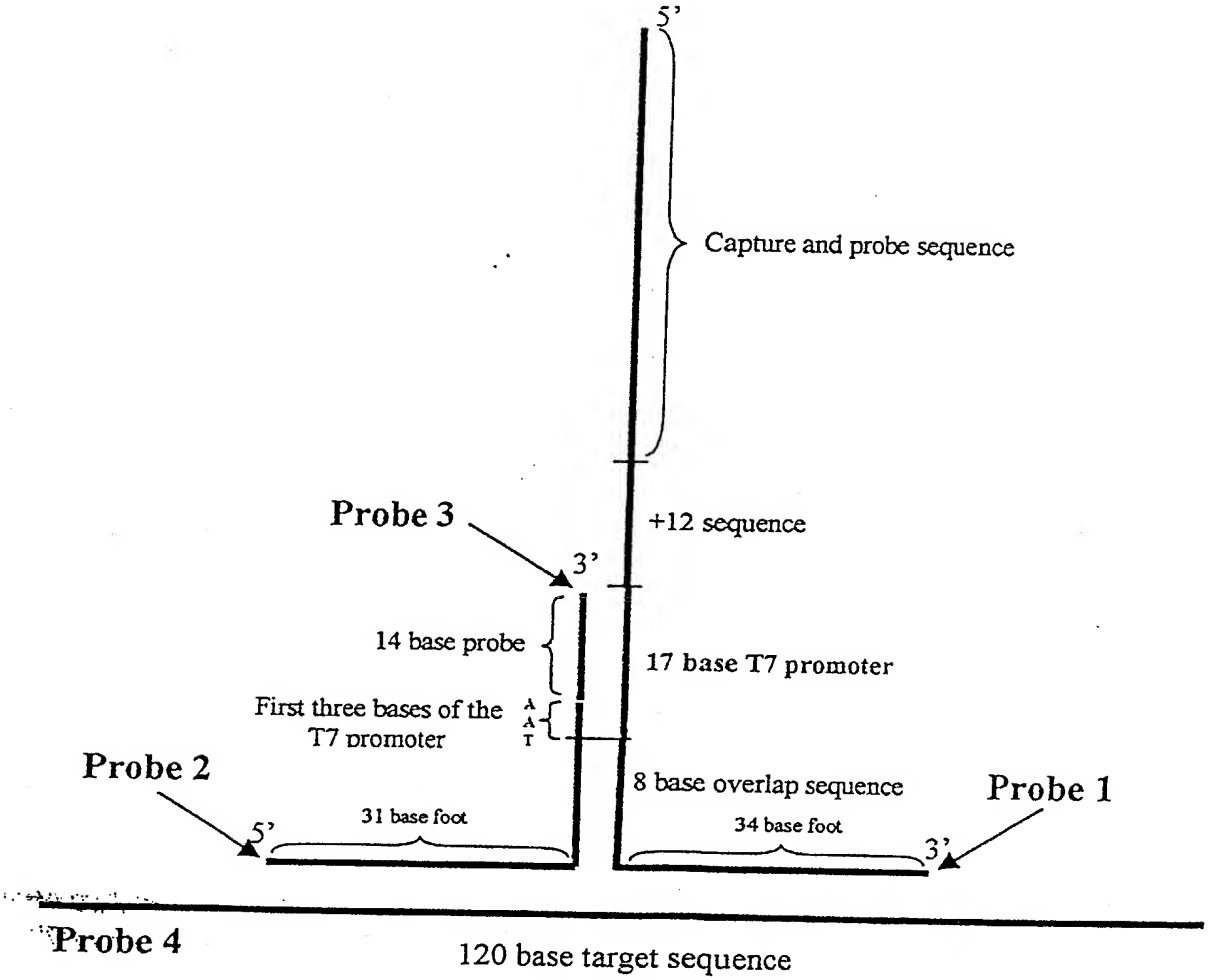
Capture Probe

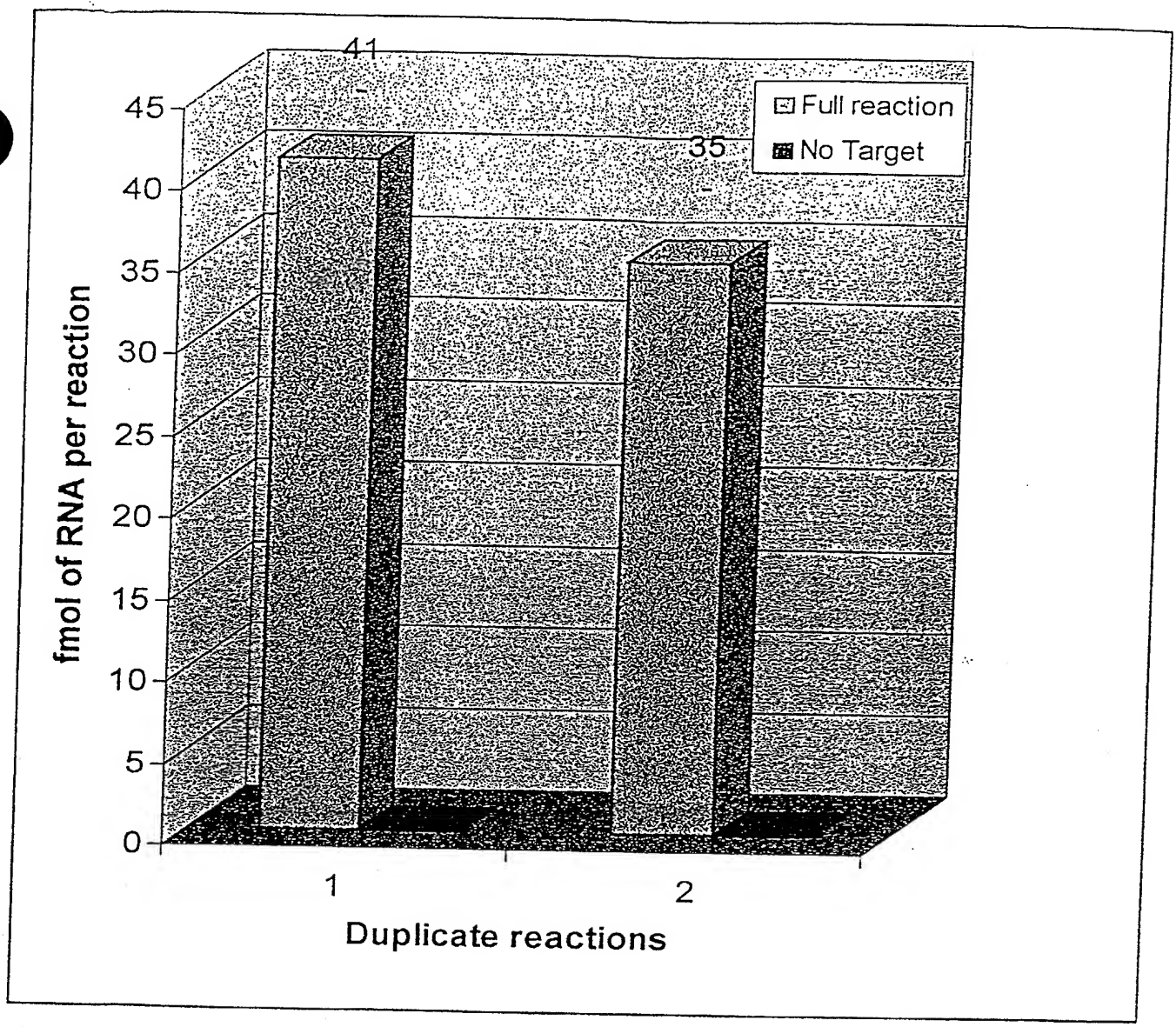
5' TCTGCTGCCTGCTTGTCTGCGTTCT 3'

Detection probe (Wallac-AP)

5' GGATATCACCCG 3' (3' alkaline phosphatase labelled)

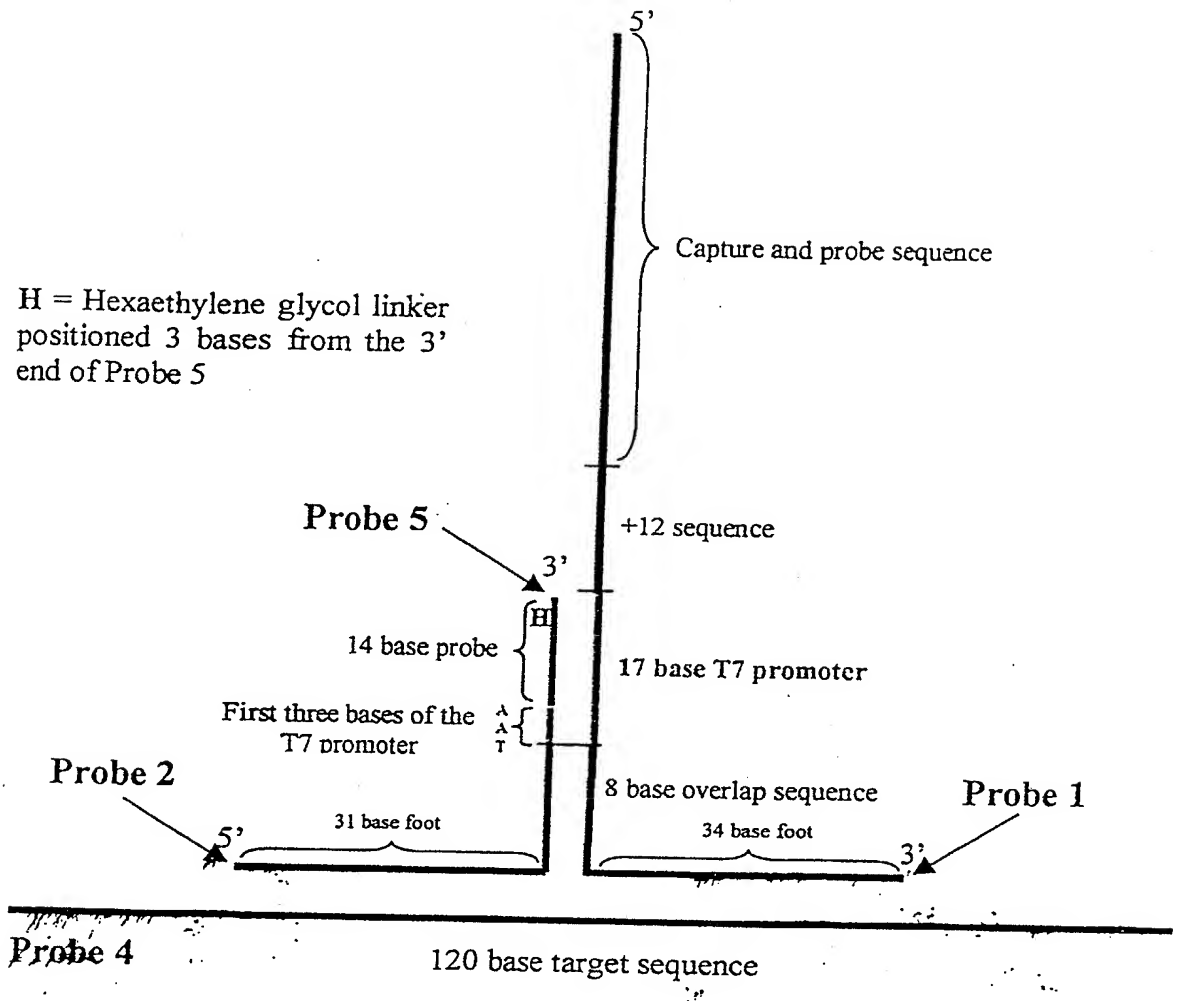
Fig. 1

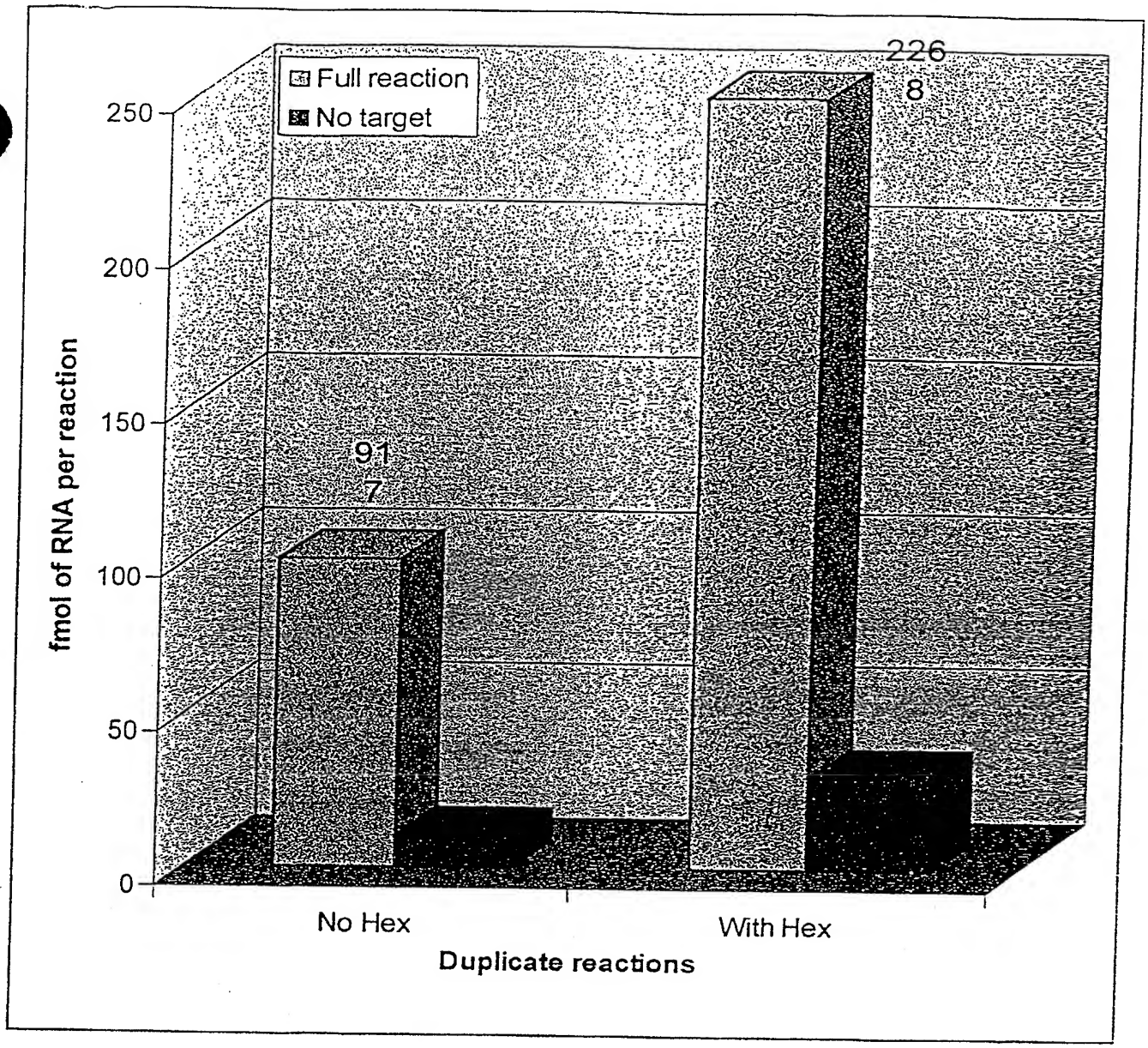

5'
Capture and probe sequence
Probe 3
+12 sequence
3'
14 base probe
17 base T7 promoter
First three bases of the T7 promoter
A
A
T
Probe 2
8 base overlap sequence
Probe 1
31 base foot
34 base foot
5'
3'
Probe 4
120 base target sequence



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

Fig. 3


5'
Capture and probe sequence
H = Hexaethylene glycol linker positioned 3 bases from the 3' end of Probe 5
Probe 5
+12 sequence
3'
H
14 base probe
17 base T7 promoter
First three bases of the T7 promoter
A
A
T
Probe 2
8 base overlap sequence
Probe 1
5'
31 base foot
34 base foot
3'
Probe 4
120 base target sequence



The numbers located above the columns represent the amplification factor (top number) and signal:noise ratio (bottom number).

Figure 5